MANUAL

Technology: HTRF™ Cytokine

HTRF High Performance Human TNF alpha Kit

Part number:	62HTNFAV2PEG	62HTNFAV2PEH
Test size	500 tests	10,000 tests

Storage: ≤ -16°C

Version: 01 **Date:** January 2025

ASSAY PRINCIPLE

This kit is intended for the simple and rapid quantification of human TNFα in supernatant and offers a fast alternative to ELISA.

The detection principle of this kit is based on HTRF™ technology (Homogeneous Time-Resolved Fluorescence). As shown in Figure 1, TNFα is detected in a sandwich assay by using anti- TNFα antibody labeled with Europium cryptate (donor), and anti- TNFα antibody labeled with d2 (acceptor).

When the dyes are in close proximity, the excitation of the donor with a light source (laser or flash lamp) triggers a Fluorescence Resonance Energy Transfer (FRET) towards the acceptor, which in turn fluoresces at a specific wavelength (665 nm). Signal intensity is proportional to the number of antigen-antibody complexes formed and therefore to the TNFα concentration.

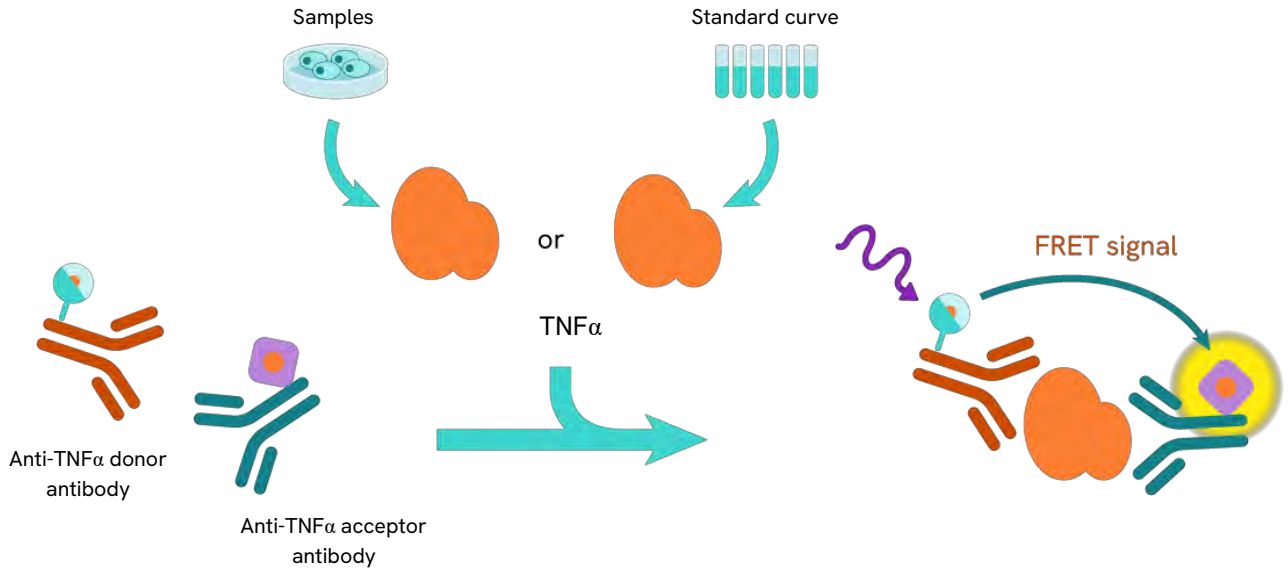
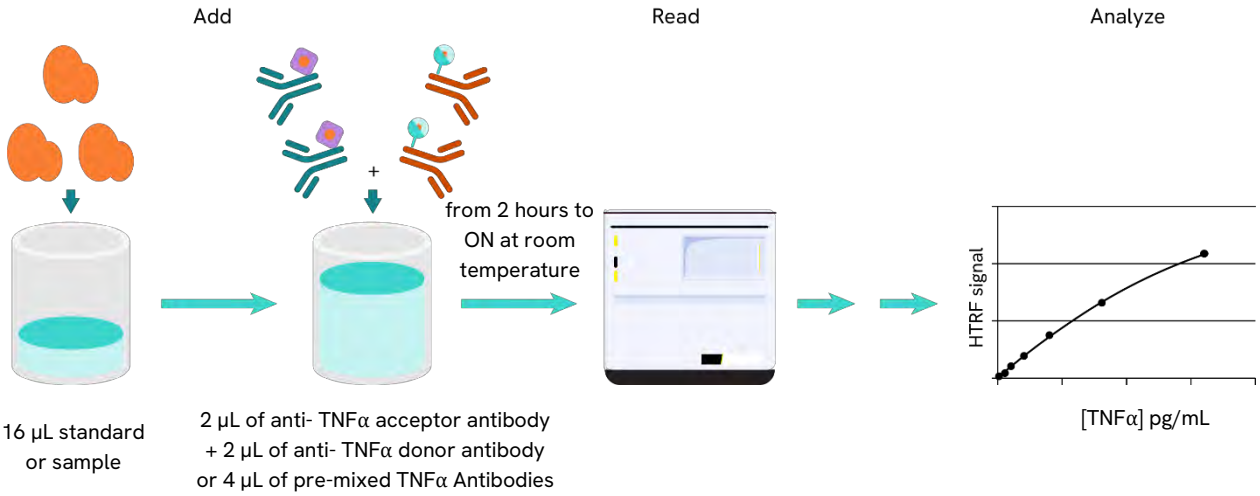


Figure 1: Principle of HTRF TNFα sandwich assay

PROTOCOL AT A GLANCE



Make sure to use the set-up for Eu Cryptate.

MATERIAL PROVIDED

KIT COMPONENTS	500 TESTS*			10,000 TESTS*		
TNFα Standard Lyophilized		green cap	2 vials		green cap	2 vials
TNFα Eu Cryptate Antibody Frozen 20X		orange cap	1 vial 50 µL		red cap	1 vial 1 mL
TNFα d2 Antibody Frozen 20X		blue cap	1 vial 50 µL		purple cap	1 vial 1 mL
Diluent** #1		Transparent cap	1 vial 20 mL		Transparent cap	2 vials 20 mL
Detection Buffer* #3 Ready-to-use**		Transparent cap	2 vials 1.5 mL		red cap	1 vial 50 mL

* When used as advised, the two available kit sizes will provide sufficient reagents for 500 tests and 10,000 tests respectively in 20 µL final volume. Assay volumes can be adjusted proportionally to run the assay in 96 or 1536 well microplates.
** Medium like cell culture medium can be an alternative to the diluent.
*** The Detection buffer is used to prepare working solutions of acceptor and donor reagents.

Purchase separately

- HTRF™-Certified Reader. Make sure the setup for Eu Cryptate is used. For a list of HTRF-compatible readers and set-up recommendations, please visit our website.
- Small volume (SV) detection microplates. Use white plate only. For more information about microplate recommendations, please visit our website.

STORAGE AND STABILITY

Kit

- Store the kit at -16°C or below.
- Under proper storage conditions, reagents are stable until the expiry date indicated on the label.
- Diluent and detection buffer are shipped frozen but can be stored at 2-8°C in your premises.

Reagents

- If lyophilized, reconstituted reagents, antibodies, and standard stock solutions may be frozen and thawed only once. To avoid freeze/thaw cycles, it is recommended to dispense remaining stock solutions into disposable plastic vials for storage at -16°C or below.
- Volume of Human TNFα standard aliquots should not be under 10 µL.

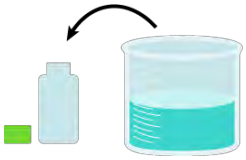
REAGENT PREPARATION

Before you begin

- It is very important to prepare reagents in the specified buffers. The use of an incorrect diluent may affect reagent stability and assay results.
- Thaw the frozen reagents at room temperature, allow them to warm up to room temperature for at least 30 mins before use.
- Before use, allow Diluent and Detection buffer to warm up at room temperature and homogenize them with a vortex.
- Antibody solutions must be prepared in individual vials and can be mixed prior to dispensing.
- TNFα standards (for standard curve) must be prepared in diluent or in the same medium as the samples.

Take care to prepare stock and working solutions according to the directions for the kit size you have purchased.

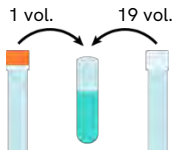
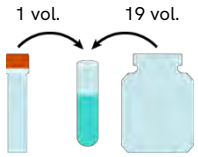
To prepare reagent stock solutions

500 TESTS		10,000 TESTS	
Anti-TNFα Eu Cryptate antibody			
Thaw the TNFα Eu Cryptate antibody. Mix gently. This 20X stock solution can be frozen and stored at -16°C or below. To avoid freeze/thaw cycles, it is recommended to dispense remaining stock solutions into disposable plastic vials for storage at -16°C or below.			Thaw the TNFα Eu Cryptate antibody. Mix gently. This 20X stock solution can be frozen and stored at -16°C or below. To avoid freeze/thaw cycles, it is recommended to dispense remaining stock solutions into disposable plastic vials for storage at -16°C or below.
Anti-TNFα d2 antibody			
Thaw the TNFα d2 antibody. Mix gently. This 20X stock solution can be frozen and stored at -16°C or below. To avoid freeze/thaw cycles, it is recommended to dispense remaining stock solutions into disposable plastic vials for storage at -16°C or below.			Thaw the TNFα d2 antibody. Mix gently. This 20X stock solution can be frozen and stored at -16°C or below. To avoid freeze/thaw cycles, it is recommended to dispense remaining stock solutions into disposable plastic vials for storage at -16°C or below.
TNFα Standard			
Reconstitute the TNFα standard with distilled water. Volume of reconstitution is indicated on the vial label. The reconstituted standard solution can be frozen and stored at -16°C or below.			Reconstitute the TNFα standard with distilled water. Volume of reconstitution is indicated on the vial label. The reconstituted standard solution can be frozen and stored at -16°C or below
Diluent			
The Diluent is ready-to-use.			
Detection buffer			
The Detection buffer is ready-to-use.			

To prepare antibody working solutions

Each well requires 2 µL of TNFα -Eu Cryptate Antibody and 2 µL of TNFα d2 Antibody.

Prepare the two antibody solutions in separate vials.

<table>
<tr><th colspan="2">500 TESTS</th><th colspan="2">10,000 TESTS</th></tr>
<tr><td colspan="4">TNFα Eu Cryptate antibody</td></tr>
<tr><td>Dilute 20-fold the 20X stock solution (thawed reagent) of TNFα Eu Cryptate antibody with Detection buffer #3: add 1 volume of Eu Cryptate antibody stock solution in 19 volumes of detection buffer (e.g. 10 µL of Eu Cryptate antibody stock solution + 190 µL of detection buffer).</td><td> 
</td><td> 
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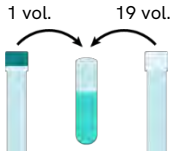
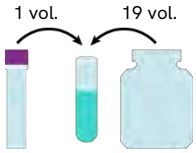
<tr><td>Dilute 20-fold the 20X stock solution (thawed reagent) of TNFα d2 antibody with Detection buffer #3: add 1 volume of d2 antibody stock solution in 19 volumes of detection buffer (e.g. 10 µL of Eu Cryptate antibody stock solution + 190 µL of detection buffer).</td><td> 
</td><td> 
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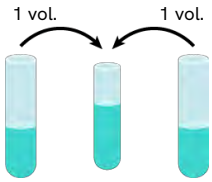
<tr><td colspan="4">Antibody Mix</td></tr>
<tr><td>It is possible to pre-mix the two ready-to-use antibody solutions just prior to dispensing the reagents by adding 1 volume of d2 antibody solution to 1 volume of Cryptate antibody solution (e.g. 1 mL of d2 antibody + 1 mL of Cryptate antibody).</td><td colspan="2"> 
</td><td>It is possible to pre-mix the two ready-to-use antibody solutions just prior to dispensing the reagents by adding 1 volume of d2 antibody solution to 1 volume of Cryptate antibody solution (e.g. 20 mL of d2 antibody + 20 mL of Cryptate antibody).</td></tr>
</table>

To prepare working standards solutions

- Each well requires 16 µL of standard.
- Dilute the standard stock solution serially with diluent #1
- If culture medium is used to dilute the standard, we recommend to supplement it with serum (2 to 10%) or BSA (0.2 to 1%) in order to avoid TNFα sticking to assay plates.
- In order to check for a potential interference effect from your own assay buffer when using the assay for the first time, we highly recommend the parallel preparation of a standard curve in your own supplemented cell culture medium and in diluent #1.
- In order to counteract any standard sticking, we recommend changing tips between each dilution.

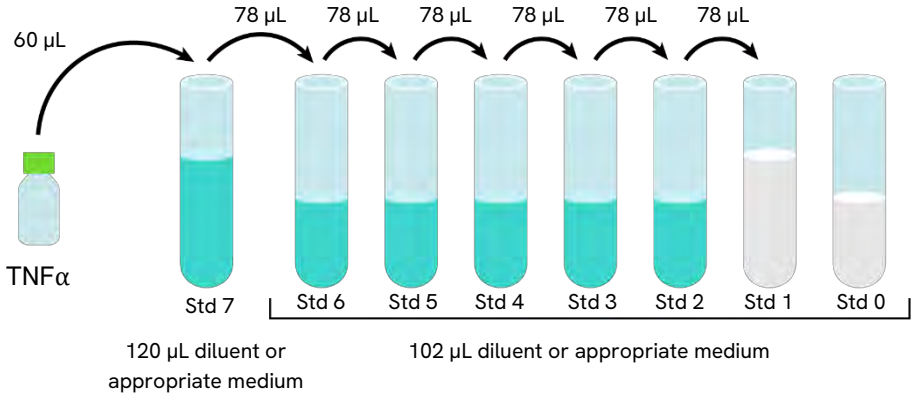
A recommended standard dilution procedure is listed and illustrated below:

Dilute the standard stock solution 3-fold with diluent; this yields the Standard Max solution (5,500 pg/mL)
Dilute the standard stock solution 3-fold with diluent #1 to prepare high standard (Std 7): e.g. take 60 µL of standard stock solution and add it to 120 µL of diluent #1. Mix gently.

Use the high standard (Std 7) to prepare the standard curve using serial dilutions as follows:

- Dispense 102 µL of diluent #1 in each vial from Std 6 to Std 0.
- Add 78 µL of standard to 102 µL of diluent #1, mix gently and repeat the serial dilution to make standard solutions: std6, std5, std4, std3, std2, std1.

This will create 7 standards for the analyte. Std 0 (Negative control) is diluent #1 or appropriate culture medium alone



STANDARD	SERIAL DILUTIONS	WORKING SOLUTIONS
Standard Stock solution	Reconstituted lyophilisate	16.5 ng/mL
Standard 7	60 µL Standard stock Solution + 120 µL diluent #1	5 500 pg/mL
Standard 6	78 µL standard 7 + 102 µL diluent #1	2391 pg/mL
Standard 5	78 µL standard 6 + 102 µL diluent #1	1040 pg/mL
Standard 4	78 µL standard 5 + 102 µL diluent #1	452 pg/mL
Standard 3	78 µL standard 4 + 102 µL diluent #1	196 pg/mL
Standard 2	78 µL standard 3 + 102 µL diluent #1	85 pg/mL
Standard 1	78 µL standard 2 + 102 µL diluent #1	37 pg/mL
Standard 0	102 µL diluent #1	0

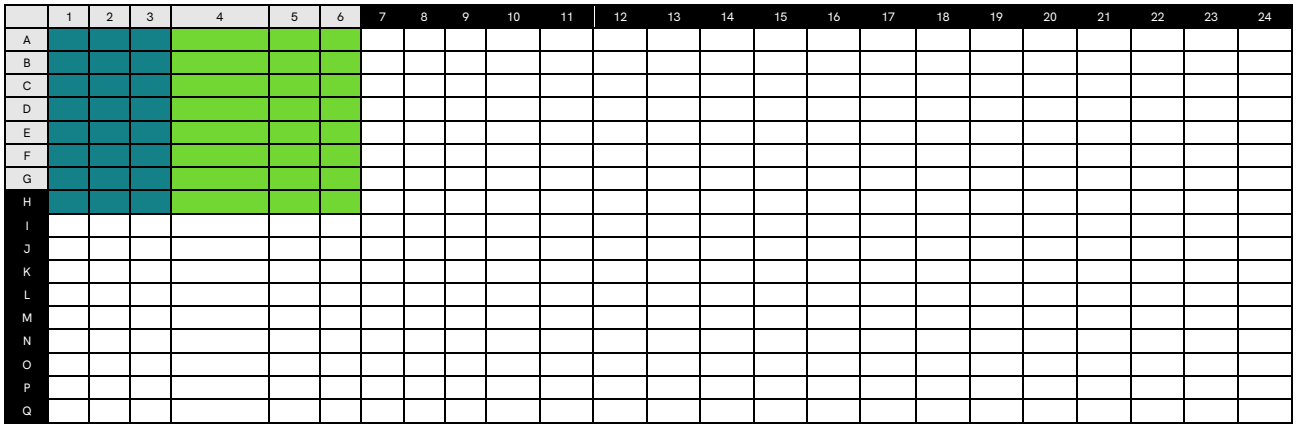
To prepare samples

- Each well requires 16 µL of sample.
- Just after their collection, put the samples at 4°C and test them immediately. For later use, samples should be dispensed into disposable plastic vials and stored at -60°C or below. Avoid multiple freeze/thaw cycles.
- Samples with a concentration above the highest standard (Std 7) must be diluted diluent or in your appropriate sample medium.
- To obtain additional information or support, please contact the HTRF technical support team.

ASSAY PROTOCOL

		STANDARD (STD 0 – STD 7)	SAMPLES
Step 1		Dispense 16 µL of each TNFα standard (Std 0 - Std 7) into each standard well	Dispense 16 µL of each sample into each sample well
Step 2		Add 2 µL of TNFα d2 antibody working solution to all wells	
Step 3		Add 2 µL of TNFα Eu Cryptate antibody working solution to all wells.	
Step 4		Seal the plate and incubate from 2 hours at RT	
Step 5		Remove the plate sealer and read on an HTRF™ compatible reader	

	1	2	3	4	5	6
A	16 µL Std 0 (Negative control) 2 µL TNFa-d2 2 µL TNFa-Eu Cryptate	Repeat Well A1	Repeat Well A1	16 µL sample 1 2 µL TNFa-d2 2 µL TNFa-Eu Cryptate	Repeat Well A4	Repeat Well A4
B	16 µL Std 1 2 µL TNFa-d2 2 µL TNFa-Eu Cryptate	Repeat Well B1	Repeat Well B1	16 µL sample 2 2 µL TNFa-d2 2 µL TNFa-Eu Cryptate	Repeat Well B4	Repeat Well B4
C	16 µL Std 2 2 µL TNFa-d2 2 µL TNFa-Eu Cryptate	Repeat Well C1	Repeat Well C1	16 µL sample 3 2 µL TNFa-d2 2 µL TNFa-Eu Cryptate	Repeat Well C4	Repeat Well C4
D	16 µL Std 3 2 µL TNFa-d2 2 µL TNFa-Eu Cryptate	Repeat Well D1	Repeat Well D1	16 µL sample... 2 µL TNFa-d2 2 µL TNFa-Eu Cryptate	Repeat Well D4	Repeat Well D4
E	16 µL Std 4 2 µL TNFa-d2 2 µL TNFa-Eu Cryptate	Repeat Well E1	Repeat Well E1	16 µL sample... 2 µL TNFa-d2 2 µL TNFa-Eu Cryptate	Repeat Well E4	Repeat Well E4
F	16 µL Std 5 2 µL TNFa-d2 2 µL TNFa-Eu Cryptate	Repeat Well F1	Repeat Well F1	16 µL sample... 2 µL TNFa-d2 2 µL TNFa-Eu Cryptate	Repeat Well F4	Repeat Well F4
G	16 µL Std 6 2 µL TNFa-d2 2 µL TNFa-Eu Cryptate	Repeat Well G1	Repeat Well G1	16 µL sample... 2 µL TNFa-d2 2 µL TNFa-Eu Cryptate	Repeat Well G4	Repeat Well G4
H	16 µL Std 7 2 µL TNFa-d2 2 µL TNFa-Eu Cryptate	Repeat Well H1	Repeat Well H1	16 µL sample... 2 µL TNFa-d2 2 µL TNFa-Eu Cryptate	Repeat Well H4	Repeat Well H4



DATA REDUCTION & INTERPRETATION

1) Calculate the ratio of the acceptor and donor emission signals for each individual well.

$$\text{Ratio} = \frac{\text{Signal 665 nm}}{\text{Signal 620 nm}} \times 10^4$$

2) Calculate the % CVs. The mean and standard deviation can then be worked out from ratio replicates.

$$\text{CV (\%)} = \frac{\text{Standard deviation}}{\text{Mean Ratio}} \times 100$$

For more information about data reduction, please visit our website.

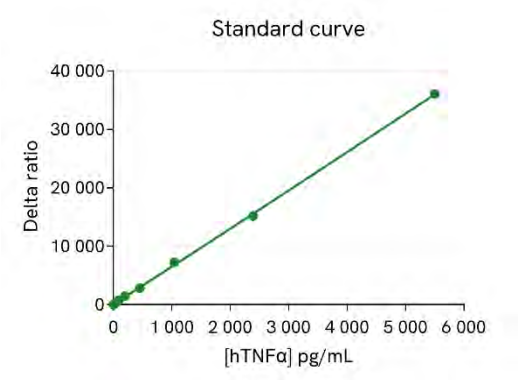
RESULTS

This data must not be substituted for the data obtained in the laboratory, and should be considered only as an example.

Results may vary from one HTRF™ compatible reader to another.

Standard curve fitting with the 4 Parameter Logistic (4PL with $1/Y^2$) model

		Ratio (1)	CV% (2)
Standard 0	Negative control	1171	3,1%
Standard 1	37 pg/mL	1407	3,4%
Standard 2	85 pg/mL	1878	8,0%
Standard 3	196 pg/mL	2610	6,7%
Standard 4	452 pg/mL	4273	3,6%
Standard 5	1 040 pg/mL	8438	2,6%
Standard 6	2391 pg/mL	16353	2,9%
Standard 7	5 500 pg/mL	37235	0,8%



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